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1 Please give the title of the invention: DNA MOLECULES AND CONSTRUCTS AND THEIR USE IN THE TREATMENT OF MASTITIS.

### ❷ Applicant's details

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# DNA Molecules and Constructs and their Use in the Treatment of Mastitis.

The present invention relates to DNA sequences, expression cassettes and DNA constructs for use in therapy, specifically in gene therapy for the treatment of mastitis. Also included are pharmaceutical and veterinary compositions containing the constructs, and cells which have been transformed with the DNA and which are suitable for implantation into a host mammal.

Bovine, caprine, ovine and porcine mastitis remain some of the most costly diseases in animal agriculture. Mastitis represents a significant economic loss to the diary industry, approximately 70 to 80 percent of which can be attributed to a decrease in milk production (Lightner et al., 1988, J. Am. Vet. Med. Assoc. 192:1410). Many infective agents have been implicated as causes of mastitis, including different agents in different species such as cows, sheep, goats and pigs.

Despite significant progress in mastitis control due to widespread adoption of postmilking teat antiseptisis, many herds continue to be plagued by this disease (Smith, 1983, J. Dairy Sci. 66:1790). A variety of different procedures have been described and used to cure mastitis caused by bacteria and yeasts. These procedures include the systemic immunization of the infected animals with whole or partial protein extracts of the infective agents in order to stimulate the immune response of the treated animal to these agents. Antibodies generally produced in

this way act against a membrance protein, a binding protein or a toxin secreted by the microorganisms. Hence these antibodies act as anti-adhesive, antitoxin, neutralising or opsonic molecules (Nordhaug et al., 1994, J Dairy Sci: 77:1267 and 1276). Nevertheless, the blood-milk barrier precents all but a very small proportion of circulation IgG antibodies from reaching mammary secretion during lactation (Colditz et al., 1985, Aust. Vet. J. 62:145).

Other procedures have been carried out in order to stimulate the diapedesis and phagocytosis of infective agents by leukocytes, more particularly polymorphonuclear neutrophils and macrophages. The stimulating molecules, which have been administered by intrammary injection, include cytokines, interleukin-1ß, interleukin 2 (Quinoga et al., 1993, J. Dairy Sci. 76:2913), interferon-γ, tumor necrosis factor-α (Sanchez et al., 1994, J. Dairy Sci. 77:1251).

The most widely used procedure to cure mastitis is the intramammary injection of antibiotics. However, this approach inflicts a lot of side effects to the animal and the milk must be discarded during the treatment period.

The use of lysostaphin in the treatment of *Staphylococcus aureus* infections of the mouse mammary gland has been described (Bramley et al., Res. In Vet. Sci, 1990, 49, 120-121. Furthermore, the use of recombinant lysostaphin, a bactericial enzyme as a mastitis therapeutic has been considered (Olhamet et al.,

J. Dairy Sci, 1991, 74, 4175-4182).

Unfortunately, all currently available procedures are very-temporary and consequently relatively inefficient. None of the gram positive bacterial are entirely eliminated from the udder after treatments with antibiotics. An effective mastitis therapy for the lactating dairy cow remains a major unfulfilled need. Because current therapy is only moderately effective and is costly because of discarded milk and culling infected animals, dry period treatment only has been the adopted herd management practice of choice.

Mammary-gland promoters have been used in transgenic animals to limit transgene expression to the mammary gland. Gene therapy techniques to target just one organ for introduction of a foreign gene have also been demonstrated. Most efforts toward postnatal gene therapy have relied on new genetic information into tissues: target cells are removed from the body, infected with viral vectors carrying the new genetic information, and reimplanted into the body (Ledley et al., 1989, Biotechniques 6:608). For some applications, direct introduction of genes into tissues *in vivo*, with or without the use of viral sectors, would be useful. Direct *in vivo* gene transfer into postnatal animals has been achieved with formulas of DNA encapsulated in liposomes, DNA entrapped in proteoliposomes containing viral envelope receptor proteins (Nicolau et al., PNAS USA 80:1968), calcium phosphate-coprecipitated DNA (Benvenisty et al., 1986, PNAS USA 83:9551), and DNA

coupled to a polylysine-glcoprotein carrier complex (Wu and Wu, 1988, J. Biol. Chem. 263:14621). *In vivo* infectivity of cloned viral DNA sequences after direct intrahepatic injection with or without formulation of calcium phosphate coprecipitates has also been described (Seeger et al., 2984, PNAS USA 81:5849). With the use of cationic lipid vesicles (Felgner et al., 1989, PNAS USA 84:7413), mRNA sequences containing elements that enhance stability can be efficiently translated in tissue culture cells (Malone et al., 1989, PNAS USA 86:6077) and in *Xenopus* *laevis* embryos (Malone, 1989, Focus 11:61).

Direct transfer of the hGH gene into the mammary gland using replication-defective retrovirus vectors has been described (Archer et al., Proc. Nat. Acad. Sci. USA, 1994, 91, 6840-6844.

However, these approaches have not previously been tried in the context of the prophylaxis and treatment of mastitis.

The present applicants have found that expression of proteins in mammary glands over an extended time period is possible and that a gene therapy approach to the problem of mastitis is feasible. Integration of a gene which encodes a therapeutic protein or polypeptide into mammary gland tissue would allow, for example, for the elimination of infective microorganisms by genetic therapy. In addition, gene therapy of the mastitic gland eliminates all side effects of other procedures, also enabling an inserted gene to synthesize permanently and

inductively or constitutively an effective amount of its therapeutic protein product. A gene therapy approach would be a much more specific and effective system of mast~~i~~tic treatment than is currently available.

Hence the present invention provides a recombinant DNA which comprises a nucleotide sequence which encodes a protein or polypeptide which is useful in the prophylaxis or treatment of mastitis, and at least one regulatory control element which allows for expression of said nucleotide sequence in a mammary gland.

Suitable regulatory control elements include transcription and translation regulatory sequences. Transcription and translation regulatory sequences are those DNA sequences necessary for efficient expression of the product. In general, such regulatory elements can be operably linked to any nucleotide sequence to control the expression of the sequence, the entire unit being referred to as the "expression cassette". Hence the invention further provides an expression cassette containing the above-mentioned recombinant DNA.

An expression cassette will typically contain, in addition to the coding nucleotide sequence, a promoter region, a translation initiation site and a translation termination sequence.

Unique endonuclease restriction sites may also be included at the end of an expression cassette to allow the cassette to be easily inserted or removed when

creating DNA constructs for use in transformations as is known in the art.

In particular the invention provides a DNA construct designed to express a protein or polypeptide which is useful in the prophylaxis or treatment of mastitis after after insertion into the mammary gland. Suitably the DNA construct comprises an inducible or constitutive promoter which is linked to a coding nucleotide sequence or gene and thereby expresses a therapeutic or protective protein which acts against infectious or potentially infectious microorganisms responsible for the mastitis of mammals.

Such DNA constructs can be administered to both lactating or non-lactating animals for the prophylaxis or treatment or mastitis. Hence the invention further provides a method for the prophylaxis or treatment of mastitis which comprises transformation of mammary gland tissue with a DNA construct as described above.

Transformation of mammary gland tissue generally requires that the DNA be physically placed within the host gland. Current transformation procedures use a variety of techniques to introduce naked DNA into a cell and these can be used to transform a mammary gland. For example, the DNA can be injected directly into glands through the use of syringe. Alternatively, high velocity ballistics can be used to propel small DNA associated particles into the gland through an udder's skin incision.

The DNA can also be introduced into a mammary gland by

insertion of other entities which contain DNA. These entities include minicells, cells (e.g. fibroblasts, adipocytes, Mac-T cells, myoepithelial cells, mammary carcinoma cells, kidney cells), liposomes (e.g. natural or synthetic lipid vehicles, cationic liposomes) or other fusible lipid-surfaced bodies. The entities are transformed *in vitro* prior to insertion using the above-described DNA constructs.

Thus the invention also provides a cell which has been transformed using a DNA construct as described above. Examples of such cells include Mac-T cells. Genetically transformed cells of this type are suitable for reimplantation into a mammary gland to produce the desired proteins or polypeptides.

Furthermore the invention provides a liposome which incorporates the above-described DNA construct.

Introduction of the naked or complexed DNA constructs into the mammary gland can be performed by direct injection through a skin incision of the udder or through the teat canal.

Where appropriate, the DNA construct is administered in the form of a pharmaceutically or veterinary acceptable composition in combination with a suitable carrier or diluent. Suitable carriers are liquid carriers such as water, salts buffered saline or any other physiological solutions. These compositions form a further aspect of the invention.

The protein or polypeptides produced should be effective prophylaxis or treatment of mastitis. Such proteins or polypeptides include mucolytic proteins such as enzymes, antibiotics, antibodies, cytokines, tumour necrosis factors as well as proteins which can induce an immune response to infective or potentially infective agents and those which activate polymorphonuclear neutrophils, or macrophages.

In a preferred embodiment, the invention provides a recombinant DNA sequence which comprises a nucleotide sequence which encodes a lytic protein or antibody under the control of a mammary gland specific promoter, or any ubiquitous or inducible non mammary promoter.

The invention is particularly applicable for the treatment of farm animals: bovine, caprine, ovine, and porcine, but can concern also lower mammals or lower milk producers: rabbit, camel and bison. The invention can also be used in humans to eliminate particularly most staphylococci.

The invention will now be particularly described by way of example with reference to the accompanying diagrammatic drawings in which

| | |
|---|---|
| Fig 1 | is schematic representation of DNA construct in accordance with this invention. |
| Fig 2 | illustrates examples of DNA constructs in |

accordance with this invention.

Fig 3 illustrates the rate of synthesis of human growth hormone in milk's sheep after injection of cationic liposome-DNA complex into the mammary gland.

In accordance with a preferred embodiment of the invention, a mammary gland of a mammal is transfected with a recombinant DNA sequence which comprises a nucleotide sequence which encodes a molecule such as a protein or polypeptide which is useful in the prophylaxis or treatment of mastitis. Suitable the molecule encoded is one which will be relayed to the udder, there eliminate or protect against the infective or potentially infective microbial agents.

Suitably the coding nucleotide sequence will be under the regulatory control of a promoter. Where the coding nucleotide sequence comprises a gene for said protein or polypeptide, it will include its own promoter which will primarily direct gene expression. However, different and/or additional promoters and promoter sequence elements may be included in the recombinant DNA sequence as well as other DNA regulatory elements in order to ensure efficient expression of the gene product.

For instance, promoter sequence elements which may be used include the TATA box consensus sequence (TATAAT), which is usually arranged 20 to 30 base pairs (bp) upstream of the transcription start site. In most

instances the TATA box is required for accurate transcription initiation. By convention, the transcription start site is designated +1. Sequences expending in the 5' (upstream) direction are given negative numbers and sequences extending in the 3' (downstream) direction are given positive numbers.

Promoters can be either constitutive or inducible. A constitutive promoter controls transcription of a gene at a constant rate during the life of a cell, whereas an inducible promoter's activity fluctuates as determined by the presence (or absence) of a specific inducer. The regulatory elements of an inducible promoter are usually located further upstream of the transcriptional start site than the TATA box. Ideally, for experimental purposes, an inducible promoter should possess each of the following properties: a low to nonexistent basal level of expression in the absence of inducer, a high level of expression in the presence of inducer, and an induction scheme that does not otherwise alter the physiology of the cells.

The basal transcriptional activity of all promoters can be increased by the presence of "enchancer" sequences. Although the mechanism is unclear, certain defined enhancer regulatory sequences are known, to those familiar with the art, to increase a promoter's transcription rate when the sequence is brought in proximity with promoter.

Preferred constitutive promoters are those which are

naturally active in a tissue specific manner in the mammary gland. Suitable mammary gland specific promoters include promoters derived from the genes which encode encode casein, lactoglobulin, lactoferrin, lactalbumin, lysozyme or whey acidic proteins (WAP).

Preferably the promoter originates from domestic animals, bovine, caprine, ovine or porcine species. Alternatively, mammary promoters can originate from smaller animals, lagomorphs, rodents, felines or canines as well as from wild mammals such as moose, bison, roe deer or caribou. Other constitutive promoters like cytoplasmic β-actin or ubiquitine can be used. Viral or retroviral promoters can be used also, like Cytomegalaovirus (CMV), Simian virus 40 (SV40) or mouse mammary tumor virus (MMTV, which is additionally inducible).

Inducible promoters include any promoter capable of increasing the amount of gene product produced, by a given gene, in response to exposure to an inducer. Inducible promoters are known to those familiar with the art and a variety exist that could conceivably be used to drive expressing of the protective or curative molecule's gene.

Two preferred inducible promoters are the heat shock promoter (HST) and the glucocorticoid system. Promoters regulated by heat shock, such as the promoter normally associated with the gene encoding the 70-kDa heat shock protein, can increase expression several-fold after

exposure to elevated temperatures. The heat shock promoter could be used as an environmentally inducible promoter for controlling transcription of the coding nucleotide sequence or gene. The glucocorticoid system also functions well in triggering the expression of nucleotide sequences or genes. The system consists of a gene encoding glucocorticoid receptor protein (GR) which in the presence of a steroid hormone forms a complex with the hormones. This complex then binds to a short nucleotide sequence (26 bp) named the glucocorticoid response element (GRE), and this binding activates the expression of linked genes. The glucocorticoid system can be included in the DNA transformation construct as a means to induce protective or curative molecule's expression. Once the constructs have been inserted the systemic steroid hormone or glucocorticoid will associate with the constitutively produced GR protein to bind to the GRE elements, thus stimulating expression of the protein or polypeptide which is useful in the prophylaxis or treatment of mastitis.

The DNA construct of the invention should allow production of the desired protein or polypeptide product in the mammary gland in an amount sufficient to produce the desired prophylactic or therapeutic effect. This will vary depending upon the type of agent being produced and the effect required, the seriousness of the condition and the nature of the patient. However, it is preferable that the agents are expressed in the mammary glands in concentrations at which they are usually employed in the treatment or prophylaxis of mastitis.

Particular examples nucleotide sequences which can be incorporated into the DNA sequence of the invention include those which encode the following polypeptides:

1. Enzymes (e.g muclolytic proteins such as lysostaphin, mucloysins);
2. Antibodies (eg. Antibody anti-hemolysins, -leucocidin, -protein A, -collagen, -fibronectin binding protein, -laminim, -α-toxin, -β-toxin) or opsonic antibodies
3. Cytokines such as interleukines or interferons;
4. Tumor necrosis factors
5. Antibiotics (which are suitable used in combination with an inducible promoter).

A particularly preferred embodiment of the invention relates to DNA sequences which encode a neutralizing lytic of opsonic molecules. Preferably, the gene coding for a mucolytic protein (e.g. lysostaphin, mucolysins) can be used to eliminate the Gram positive bacteria (mostly coccus).

For example, *Straphylococcus aureus* is one of the primary etiological agents of bovine mastitis and a major cause of economic loss to the dairy industry. A recombinant protein such as recombinant lysostaphin (rLYS) with bactericidal activity against *S. aureus* would be extremely useful therapy for the veterinarian. If rLYS was as effective as antibiotics, natural proteolysis and inactivation in the milk of rLYS, as well as inactivation during ingestion by the consumer, would

potentially minimize any concerns associated with residues in the milk.

Other microorganisms which can be responsible for the mastitis and which can treated with the DNA constructs of the invention include:

In cattle *Streptococcus agalactiae, Str. Ube, Str. Zooepidemicus, Str. Dysgalactiae, Str. Faecalis* and *Str. pneumoniae, Straphylococcus aureus, Escherichia coli, Klebsiella spp., Corynebacterium pyogenes, Cor. bovis, Mycobacterium tuberculosis, Mycobacterium spp., Bacillus cereus, Pasteurella multocide, Pseudomonas pyocyaneus, Sphaerophorus necrophorus, Serratia marcescens, Mycoplasma spp., Nocardia spp.*, a fungus *Trichosoporon spp.*, yeasts *Candida sp., Crptococcus neoformans, Saccharomyces*, and *Torulopsis spp.*

In sheep: *Pasteurella haemolytica, Staph. Aureus, Actimobacillus lignieresi, E. coli, Str. Uberis* and *Str. agalactiae*, and *Cor pseudotuberculosis*.

In goats: *Str. agalactiae, Str. Dysgalactiae, Str. Pyogenes*, and *Staph. aureus*.

In pigs: *Aerobacter aerogenes, E. coli, Klebsiella spp., Pseudomonas aeruginosa*, coagulase-positive staphylococci, *Str. agalactiae, Str.*

*dysgalactiae,* and *Str. uberis.*

In horses: *Corynebacterium pseudotuberculosis, Str. Zooepidemicus,* and *Str. equi.*

The following examples are intended to illustrate but not limit the extent of the invention.

## EXAMPLE 1

**Long-term persistence of plasmid DNA and foreign expression in sheep mammary glands.**

We show here that an injection of pure DNA complexed to cationic liposomes directly into a sheep's mammary gland results in significant expression of reporter gene within the gland.

*Preparation of plasmid-liposome mixture.*

Plasmid pCR3 (In-Vitrogen) was used as mammalian expression vector. After PCR amplification, the human growth hormone (hGH) cDNA was inserted into pCR3. This resulted in plasmid construct pCR3. Plasmid-lipofectamine (BRL) mixture was prepared as described by the manufacturer (GibcoBRL). Briefly, 50 ug of pCR3-hGH suspended in 500 μl sterile phosphate buffered saline (PBS), was mixed to 100 μl of Lipofectamine also previously diluted into 500 μl of PBS, and kept at room temperature at 1 hour.

*Infusion of the plasmid-liposome complexes into sheep mammary gland.*

The circular pCR3-hGH plasmid-lipfectamin mixture was loaded into a glass syringe. Just after dropping, by using a 20-gauge needle, the DNA-liposome complex was infused directly through the udder's skin into the mammary parenchyma. One ml was injected into the right quarter of two ewes. The milk of the left glands was used as negative controls.

*Analysis of sheep milk.*

Sheep were milked once daily by hand with the milk kept at -80°C until analysed. The amount of hGH was measured by immunoassay (Immunocorp) after determining that the milk did not affect the accuracy of the assay. Aliquots (100 ul) of milk samples were analysed.

**RESULTS**

hGH synthesized by injecting pCR3-hGH into the mammary gland was detected all along the lactating period, meaning about 60 days, as illustrated in Figure 3. The concentration of hGH in the sheep's milk was relatively high during the first 5 days. At that time it was of 300 to 400 ng/ml (± 43 ng/ml). hGH concentrations in the milk from the left (control) gland was from 10 to 15 ng/ml for the two sheep everyday of the experiment. No important differences of concentration of hGH in milk samples were found between each ewes.

These results demonstrate that expression from plasmid DNA can persist in a sheep's mammary gland for at least 60 days. The unprecedented ability of plasmid DNA to stably express a foreign gene in a mammary gland throughout the lactating period of a sheep has important implications for gene therapy. The stable expression of circular plasmid DNA suggest that foreign acceleration or by viral transduction should also be stably maintained.

## EXAMPLE 2

**Human growth hormone (hGH) secretion in goats' milk after direct transfer of the hGH gene into the mammary gland.**

An alternative route of introducing genes into the mammary parenchyma is through expansion of gene therapy techniques. In this study two Gibbon ape leukemis virus (GaLV) pseudotype retroviral vectors were used to transfer reporter genes into a goat's mammary secretory epithelial cells *in vitro* and *in vivo*.

*Cells and tissue culture.* MDBKs, a bovine kidney cell line and Mac-T cells, a bovine mammary epithelial cell line were used. Retroviral packaging cell lines used (φCre, PA317, and PG13/LNc8) were acquired from ATCC. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with gentamycin (54 mg/ml) and 10% fetal calf serum, 37°C with 5%$CO^2$ /95% air.

*Establishment of producer cell lines.* A construct carrying

the JR-gal neo- (Wang et al., 1991, Cancer Res. 51:2642) was transfected into the ecotropic packaging cell line φCre by particle bombardment at 1 *vg* of DNA per mg of gold beads. Two days after bombardment, the supernatant was removed from these cells and centifruged, and after the addition of Plybrene at 4 μg/ml, the retroviral solution was used to infect both amphotropic and GaLV pseudotype packaging cell lines. A plasmid carrying the retrovirus vector, MFG-hGH was contransfected with pSV2neo at a ratio of 50:1 via particle bombardment into PA317s and PG13/LN c8s. Packaging cells producing retrovirus containing the hGH gene were selected by G418 resistance (400 *vg*/ml).

*Virus producing cells.* The PG13/LN c8 clones that yielded the highest levels of hGH produced from the target cell lines were chosen for the infusions into a goat's mammary glands. Each clone was passed three times into 200 100 mm-plates. Cellular supernatant was collected over a 3-day period, concentrated, and resuspended in DMDM with gentamycin.

*Induction of cell division and lactation of goats.* Two 2-year-old (goats 1 and 2) and two 1-year-old (goats 3 and 4) virgin Saanen-crossbred goats were treated with exogenous steroids i.m. over a 14-day interval to induce mammogenisis and subsequent lactation (Fowler et al., 1991, J. Dairy Sci. 58:151).

*Infusion of viral stocks into a goat's mammary glands.* Polybrene was added to concentrated PG13/LN c8 MFG-hGH

viral stock at 80 $\mu g/ml$ and loaded into a syringe. By using a 22-gauge stub adaptor, the retrovirusis were infused up the right mammary teat on days 3, 5, 7, 9, 11, and 13 of the hormonal regimen for goats 1, 2, and 4 and goat 3 received infusions on days 3, 5, 7, 9, 10, and 13. The amount of viral solution was different for each animal, ranging from 8 to 20 ml, and was determined by the interal capacity of the gland. The left gland served as the intraanimal control and was infused with DMEM containing genramycin. Retroviral stock used for the infusions was then assayed on several cell lines.

*Analysis of goat's milk.* Goats were milked twice daily by hand with the morning milk kept at -80° until analysed. The amount of hGH was measured by immunoassay after determining that the milk did not affect the accuracy of the assay. Aliquots (5 vl) of milk samples diluted 1:10 in double distilled water were also analysed by SDS/PAGE on 14% gels stained with Coomassie blue. The protein concentration of the milk samples was determined by using BCA (Pierce et al., 1977, Anal. Biochem, 81:478).

**RESULTS**

*Vector production of packaging cell lines.* The concentration of hGH in the medium remved from Mac-T and MDBK cells 2 days after infection with retrovirus packaged by PG13/LN c8 clone 6 was 192 and 3.8 ng/ml, respectively. Twenty-eight days after infection, hGH levels from these cells were 119.3 and 4.5 ng/ml, indicating that the provirus LTR was still functioning 4

weeks after infection.

*Infusion of viral stocks into the mammary glands of goats.* Viral stock infused on day 13 for goats 1 and 2 was found to contain hGH at 224 ng/ml, indication that the PG13/LN c8 packaging cell were also producing hGH.

*Analysis of goat milk.* Lactation commenced on day 14 of the hormonal regimen, 24 hr after the last viral infusion. Milk appeared normal throughout the lactations. The volume of milk obtained from each udder half was approximately 150 ml on the first day of lactation for goats 1 and 2 but only 10 ml for goat 3, and 35 ml for goat 4. Milk volume produced by each gland for all four goats increased daily. The levels of hGH were determined by immunoassay with unique hGH secretion patterns for each animal. In goat 1, concentration of hGH dropped steadily until day 9 of lactation when it levelled at 3-5 ng/ml, whereas goats had a more precipitous decrease in measured hGH from day 1 to day 2 of lactation, though the animal's production of hGH stabilized at 2-3 ng/ml around day 10. Milking was stopped on day 15 of lactation for goats 1 and 2. Levels of hGH in the milk of goat 3 dropped dramatically from day 1 to 2 of lactation and then increased from day 8 to day 9 where it remained at 23 ng/ml until day 16 when it began to fall again. Goat 4, in which prostaglandkin E2 was infused at the end of the remaining 19-day lactation after a decline on the first 2 days. In addition, goat 4 was still secreting hGH at 5 ng/ml after 28 days. hGH concentrations in the milk from the left (control) gland

ranged from 0.0 to 0.6 ng/ml for the four goats at all evaluated times. These numbers are at the detection level of the assay and correlate with ones measured in two other lactating goats that had no exposure to retrovirus. The total production of hGH in the four animals ranged from 0.3 to 2 ug/day.

If the hGH gene had been stably incorporated into the stem-cell population, it would have been expected that the goats would also secrete hGH in a second lactation after the gland had undergone involution. A second lactation was induced in two of the goats, and though goat 1 did not produce hGH, goat 2 began secreting detectable amounts of hGH starting on day 5 from the right (infused) gland and during the subsequent 10 days hGH concentrations varied from 0.4 to 2.3 ng/ml. Milk from the left control gland during this lactation always had no detectable levels of hGH.

SDS/PAGE of goat's milk sampled throughout the period of collection showed no consistent differences in the protein profiles from the retroviral-infused right glands, the control left glands, and a goat not exposed to the retrovirus. Protein concentrations measured by BCA of the milk with hGH were not statistically different from the control milk, thus production of hGH by the mammary secretory epithelial cells did not appear to affect the norma cellular protein machinery. There was an indication that the milk's proteins in the treated gland were not secreted at maximal concentration on day 1 of lactation.

## EXAMPLE 3

**Effect of lysostaphin on Staphylococcus aureus infections on the mouse's mammary gland.**

Lysostaphin is an endopeptidase produced by *Staphylococcus simulans*. It hydrolzes the pentaglycine links of the peptidoglycan of members of the genus *Staphylococcus* and consequently has little activity against other prokarkotes and none against eukaryotes. The lysostaphin gene has been cloned and expressed successfully in *Escherichia coli* and *Bacillus* species (Heath et al., 1987, FEMS Microbiology Letters 44:129; Heinrich et al., 1987, Molecular and General Ganetics 209:563; Recsei et al., 1987, PNAS USA 84:1127). The use of lysostaphin to promote lysis of Staphylococcus aureus in a variety of experimental situations is well known but the progress made in cloning and expressing the gene in other hosts raises the possibilities of producing large quantities of the enzyme relatively inexpensively. This may permit its use in vivo in new approaches to the control of staphylococcal mastitis, an economically important disease of lactating ruminants (Bramley et al., 1984, J. Dairy Res. 51;81). This experiment shows the use of a mastitis model in the lactating mouse and clearly demonstrates potent antibacterial activity of lysostaphin against *S. aureus in vivo.*

Lysostaphin (Sigma Chem.) was dissolved in skimmed milk (Oxoid) to provide a range of concentrations between 0.1 and 100 $\mu$g/ml. Controls without lyspstaphin were

included. One ml volumes of the controls and lysostaphin dilutions were inoculated with $10^8$ colony forming units (cfu) of *S. Aureus* M60. This strain produces both $\alpha$ and $\beta$ toxins and was isolated from a case of bovine mastitis. It has been extensively used in experimental infections (Anderson 1975, J. Comp. Pathol. 192:579). Lysostaphin concentrations exceeding 2 to 3 ug/ml in milk produced a 2 to 3 log $_{10}$ reduction in viable *S.aureus*, whereas 10 ug/ml in milk reduced *S. aureus* from a mean of 7.95 log $_{10}$/ml in the control to 2.0 $\log_{10}$/ml. Consequently a dose of 10 µg of lysostaphin was selected for use in vivo. Anaesthetised mice, of strain MF1, were inoculated in the upper pair of abdominal mammary glands (designated R4 and L4). Eight lactating mice were inoculated with $10^8$ cfu of *S. aureus* in 0.1 ml saline in both R4 and L4. This was followed one hour later by the infusion of 10 ug lysostaphin in 0.1 ml saline into R4 and o.1 ml saline into L4. After a further 30 minutes the mice were killed and the mammary glands were aseptically removed and homogenised in saline containing 0.1 mg/ml trypsin (Sigma Chem.) to destroy active lysostaphin. Ten fold dilutions were placed on 7 per cent calf blood agar (Oxoid Blood Agar Base Number 2), incubated at 37°C overnight and viable counts determined. In a further experiment using 20 mice a prophylactic use of lysostaphin was simulated by infusing 10 ug of lysostaphin intrammammarily, followed either immediately or after one hour by $10^3$ cfu of *S. aureus*. Control glands were infused with saline instead of lysostaphin. After 24 hours the mce were killed and dissected. Gross pathological changes were noted an viable *S. aureus* counts determined as described

above.

**RESULTS**

Infusion with 10µg lysostaphin into mammary glands previously inoculated with *S. aureus* reduced bacterial recoveries, compared to the controls, by more than 99 per cent in 30 min. This reduction was statistically significant ($t=2.56$; $P<0.02$). When 10 ug of lysostaphin was administered either immediately or one hour before *S. aureus* inoculation, recoveries after 24 hours averaged around $10^2$ viable *S. aureus* per mammary gland compared with approximately $10^9$ per mammary gland for the saline treated controls. In the latter case, the control glands showed severe pathological changes typical of acute staphylococcal mastitis in the mouse. The control glands were darker and reddened, had a brittle texture and some areas of liquefaction and haemolysis. Histological sections revealed a severe inflammation, infiltration of neutrophils and macrophages with areas of coagulative necrosis. Large numbers of staphylococci were visible. In contrast, the lysostaphin treated glands remained pale and elastic with only slight redenning around the base of the teat. Histological examination showed little or no cellular infiltration, a well preserved and functioning aleveloar structure and few cocci.

These experiments clearly demonstrate the anti-staphylococcal activity of lysostaphin in vivo. Both a therapeutic and prophylactic potential were demonstrated. The cloning of the lysostaphin gene may make it readily

available for therapeutic use at a competitive price and its relatively high specificity makes it attractive for use in food-producing animals. Furthermore, advances in transgenic technology allow the direction of the expression of transgenes to the mammary gland of ruminants (Simons et al., 1987, Nature 328:530). In general, this has been applied to the production of pharmacologically active substances for use in human medicine. However, the incorporation and expression of the lysostaphin gene in the lactating mammary gland could potentially increase the resistance of the animal to staphylococcal mastitis.

**EXAMPLE 4**

**Lysostaphin efficacy for treatment of *Staphylococcus aureus* intramammary infection.**

Cloned-derived lysostaphin was evaluated as to its bactericidal effect on *S. aureus* intrammary infections. *S. aureus* (Newbould 305) was eliminated from glands of guinea pigs 48 hrs post-infection by 125 ug of lysosotaphin in 14/16, 25 ug in 5/8, 5 ug in 5/10, 1ug in 0/1, and 0 ug in 0/3. Glands infected with *S.aureus* at 48 hours post-challenge in untreated guinea pigs persisted, however, 3/25 control glands of treated guinea pigs cleared in response to treatment of the adjacent gland.

Somatic cell/ml in guinea pig shifted from $10^4$ pre-infected glands to cell counts greater than $3 \times 10^6$

following *S. aureus* inoculation. Treatment with lysosotaphin caused a neutrophilic shift in the treated gland to levels exceeding $10^8$ accompanied by an increase in the adjacent non-treated gland but dropped sharply to pre-treatment level. The greatest response in control glands was observed in animals receiving 125 ug which corresponded to 2/25 clearance of *S. aureus* in control glands.

The leucocytic response to intrammary treatment in the cow is similar to the guinea pig model described above. Somatic cell levels increased ten-fold in *S. aureus* infected glands at the milking following treatment. Cell levels returned to pre-treatment levels or lower in subsequent milking. A rise in leukocytes alone could not account for clearance of the infection.

**EXAMPLE 5**

**Lysostaphin: Use of a recombinant bacterial enzyme as a mastitis therapeutic.**

A recombinant mucolytic protein, lysostaphin, was evaluated as a potential intramammary therapeutic for *Staphylocococcus aureus* mastitis in dairy cattle. Lysostaphin, a product of *Staphylococcus simulant*, enzymatically degrades the cell wall of *Straphylococcus aureus* and is bactericidal.

Thirty Holstein-Friesian dairy cattle in their first

lactation were infected with *Staphylococcus aureus* (Newbould 305, ATCC 29740) in all quarters. Infections were established and monitored for somatic cell counts and *Staphlococcus aureus* colony-forming units 3 weeks prior to subsequent treatment. Infected animals were injected through the teat canal with a single dose of recombinant lysostaphin (rLYS) (dose 1 to 500 mg) or after three successive p.m. milking with 100 mg of rLYS in 60 ml of sterile phosphate-buffered saline. Animals were considered cured if the milk remained free of *Staphylococcus aureus* for a total of 28 milkings after the last treatment.

**RESULTS**

Kinetic analysis of immunologically active rLYS demonstrated that a minimum bactericidal concentration was maintained in the milk for up to 72 hours at 37°C. In contrast, penicillin G retained less than 10% of its bacteriostatic activity over the same incubation time.

*Dose titration and kinetics of rLYS in the bovine mammary gland.* In order to determine the optimal effective dose to elicit long-term cures, a titration was performed in which a single dose of rLYS at concentrations of 0, 1, 10, 100, or 500 mg was administered. Untreated quarters and the 1-mg treatment failed to clear all quarters of *S. aureus*. The 10- 100- and 500- mg does transiently cleared the milk of *S. aureus* for at least one milking. In relapsed quarters, the length of  time of the milk remained clear of *S. aureus* was approximately proportional to the dose administered. Fourteen days

after treatment, two quarters were cured with the 100-mg dose and one with the 500-mg dose. Because rLYS maintains a minimal bactericidal concentration (MBC) for approximately 24 h and the experimental infections undergo a 2- to 4- days cycling, multiple infusions of 100 mg of rLYS over three consecutive milking were determined to be optimal to maintain a minimal effective dose for 3 to 5 days and to elicit cures.

The *in vivo* does titration suggested that the minimal effective therapeutic dose was 100 mg of rLYS. However, therapeutically, it would be desirable to administer multiple infusions of rLYS to maintain a minimal bactericidal activity within the milk of treated glands for one to three successive milkings. The in vivo bactericidal activity of rLYS was most effectively demonstrated by the fact that 95% of the quarters cleared the milk of detectable *S. aureus* for a minimum of one milking after the last intramammary infusion.

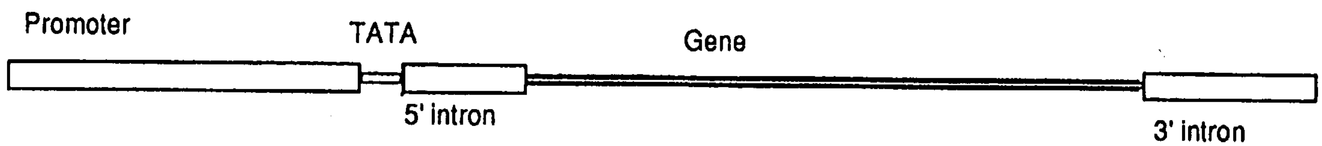


Figure 1

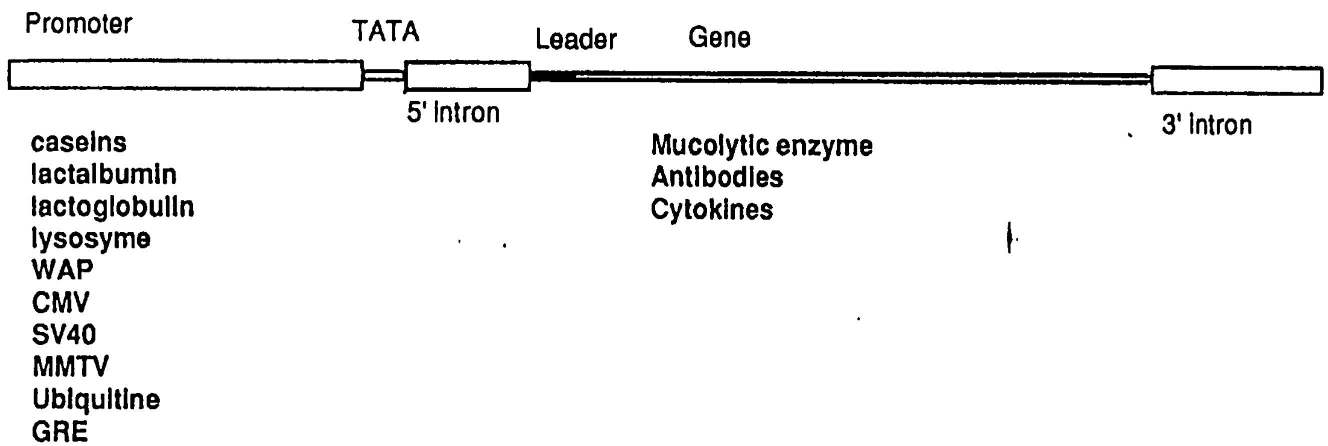
Promoter
TATA
Leader
Gene
5' Intron
3' Intron
caseins
lactalbumin
lactoglobulin
lysosyme
WAP
CMV
SV40
MMTV
Ubiquitine
GRE
Mucolytic enzyme
Antibodies
Cytokines

Figure 2

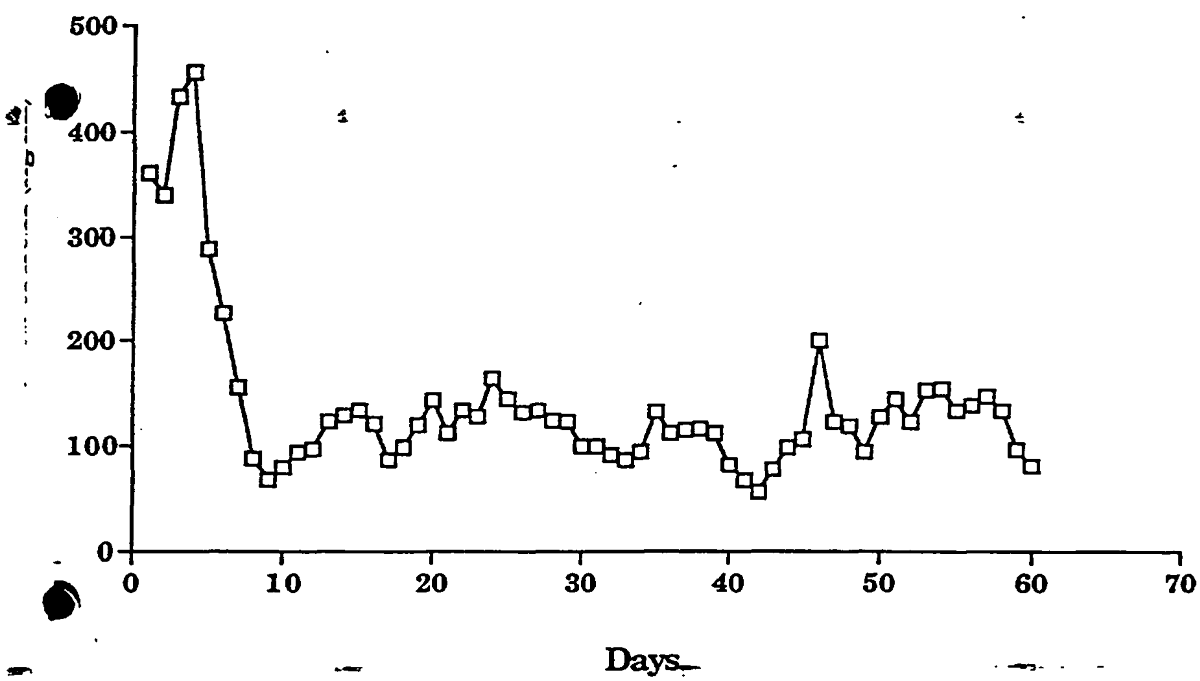


Figure 3